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Electron Microscopy of Native Cartilage Oligomeric Matrix Protein Purified from the Swarm Rat Chondrosarcoma Reveals a Five-armed Structure*

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Cartilage oligomeric matrix protein was isolated in the native state from the Swarm rat chondrosarcoma. A crucial step was its selective extraction with EDTA-containing buffer. The purified protein was subjected to electron microscopy using rotary shadowing and negative stain. The images allowed the construction of a structural model. The bouquet-like protein consists of five 28-nm-long arms containing a peripheral globular domain, a flexible strand, and a central assembly domain, where the five arms meet in a cylindrical structure.

Cartilage matrix is a composite of collagens and a variety of noncollagenous proteoglycans and glycoproteins (Paulsson and Heinegård, 1984; Heinegård and Oldberg, 1989). The functional role of this latter group of tissue constituents is still poorly understood, with the possible exceptions of link protein, which stabilizes proteoglycan aggregates (Gregory, 1973; Heinegård and Hascall, 1974; Hardingham, 1979), and fibromodulin and decorin, molecules which may regulate collagen fibrillogenesis (Hedbom and Heinegård, 1989). Other members are cartilage matrix protein (Paulsson and Heinegård, 1979, 1981) and a recently described basic 36-kDa protein (Larsson *et al.*, 1991). A further high M_r (>400,000) protein was identified by Fife and Brandt (1984) in articular and tracheal cartilage from several species and consists of disulfide-bonded subunits of apparent M_r = 116,000. It was shown to be located throughout the cartilage matrix (Fife *et al.*, 1985) and to be synthesized in organ cultures of articular cartilage (Fife *et al.*, 1986). This protein is being evaluated as a potential synovial fluid and serum marker of osteoarthritis (Fife, 1988; Fife and Brandt, 1989; Fife *et al.*, 1991). Antibodies against a bovine protein with similar or identical structural properties (Hedbom *et al.*, 1992) were used in studies of mouse limb development (Franzen *et al.*, 1987), and the antigen was shown to appear relatively late and preferentially in those peripheral regions of the cartilage model which later form the articular cartilage.

In the present study, a protein similar to the high M_r cartilage matrix glycoprotein described by Fife and Brandt (1984) and Franzen *et al.* (1987) has been isolated from the transplantable Swarm rat chondrosarcoma (Choi *et al.*, 1971). Its preferential solubility in buffers containing EDTA facilitated its purification in native form and allowed a structural analysis by molecular electron microscopy. We refer to this protein by the descriptive term COMP.[1]

MATERIALS AND METHODS

Sequential Extractions—Frozen Swarm rat chondrosarcoma tissue (5 g) was minced with a scalpel and rapidly transferred to a container with 25 ml of prechilled 10 mM Tris/HCl, pH 7.4, containing 2 mM phenylmethanesulfonyl fluoride (PMSF) and 2 mM *N*-ethylmaleimide (NEM). The tissue was suspended by brief homogenization with a Polytron homogenizer at full speed and immediately centrifuged for 20 min at 15,000 rpm and 4 °C in a Beckman JA20 rotor. The supernatant was decanted and the tissue residue was again suspended by brief homogenization in the same buffer. This extraction procedure was performed a total of four times with the low salt buffer above, four times with the same buffer containing, in addition, 10 mM EDTA, and four times with the same buffer containing 4 M guanidine HCl and 10 mM EDTA. To samples (50 μl) of all extracts, 10 μl of 1 M sodium acetate and 1.2 ml of ethanol was added, and the mixtures were incubated overnight to allow complete precipitation of protein. The pellets recovered by centrifugation were washed once by repeating the same procedure, dried, and dissolved in SDS-polyacrylamide gel electrophoresis sample buffer containing 2% 2-mercaptoethanol.

Purification of COMP—Chondrosarcoma tissue was extracted as described above for the analytical procedure but with the following modifications: 100 g of tumor tissue was used in each preparation and initially extracted three times with 500 ml of low salt buffer. The tissue residue was collected each time by centrifugation for 20 min at 8000 rpm and 4 °C in a Beckman JA10 rotor. The pellet was then extracted once with 500 ml of the EDTA-containing buffer, and the supernatant was collected. This EDTA extract was applied to an anion exchange column (39 × 2.6 cm) of DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated in 10 mM Tris/HCl, pH 7.4, containing 2 mM EDTA, 2 mM PMSF, and 2 mM NEM, at 4 °C. After washing with equilibration buffer, the column was eluted with a gradient (1000 ml) of 0–0.5 M NaCl in the same buffer. Fractions of 10 ml were collected, and samples were taken for SDS-polyacrylamide gel electrophoresis to identify COMP-containing fractions. The COMP pool was concentrated by ultrafiltration (YM10 filter, Amicon) and applied to a gel filtration column (100 × 1.6 cm) of Sepharose CL-4B (Pharmacia LKB Biotechnology) eluted with 0.15 M NaCl, 0.05 M Tris/HCl, pH 7.4, containing 2 mM EDTA, 2 mM PMSF, and 2 mM NEM. Fractions of 6 ml were collected, and those containing COMP were identified by SDS-polyacrylamide gel electrophoresis. The pool of COMP from the molecular sieve was again concentrated by ultrafiltration and finally applied to a Superose 6 HR FPLC column (30 × 1.0 cm) eluted with 0.15 M NaCl, 0.05 M Tris/HCl, pH 7.4, containing 2 mM EDTA. Fractions of 0.3 ml were collected, and pools of COMP were made after analysis of fractions by SDS-polyacrylamide gel electrophoresis.

Analytical Methods—SDS-polyacrylamide gel electrophoresis was

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[1] The abbreviations used are: COMP, cartilage oligomeric matrix protein; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; FPLC, fast protein liquid chromatography.

performed according to the protocol of Laemmli (1970), and the gels were either stained with Coomassie Brilliant Blue or subjected to electrophoretic transfer to nitrocellulose as described by Towbin *et al.* (1979). Immunoblots were developed by use of a rabbit antiserum against bovine COMP, which had been preadsorbed on a fibronectin-Sepharose column (Hedbom *et al.*, 1992), and a peroxidase-anti-rabbit IgG complex (Dakopatts, Glostrup, Denmark) using chloronaphthol as a substrate.

Glycerol spraying and rotary shadowing as well as negative staining for electron microscopy of COMP was done by previously published procedures (Engel and Furthmayr, 1987). Samples were dialyzed overnight at 4 °C against 0.2 M ammonium hydrogen carbonate and diluted with the same solvent to a final concentration of about 20 μg/ml. They were mixed with an equal volume of 80% glycerol immediately before spraying onto mica discs and shadowing with carbon/platinum on a rotating table at a 9 ° angle. For negative staining, particles (about 5 μg/ml) were adsorbed from 0.2 M ammonium hydrogen carbonate onto glow-discharged carbon films. Staining was performed with a freshly prepared uranyl formate solution at pH 4. Measurements were made on projections of the negatives onto a screen at a total magnification of 500,000.

RESULTS

Selective Extraction of COMP with EDTA-containing Buffer—Swarm rat chondrosarcoma tissue was sequentially extracted with a low salt buffer of 10 mM Tris/HCl, pH 7.4, containing 2 mM PMSF and 2 mM NEM as protease inhibitors, followed by the same buffer containing 10 mM EDTA, and finally 4 M guanidine HCl, 10 mM EDTA. Each type of extraction was repeated four times, and samples of the extracts were prepared for SDS-polyacrylamide gel electrophoresis. On a gel run under reducing conditions, it was seen that the low salt buffer extracted large amounts of highly soluble proteins (Fig. 1*a*, *extracts 1–4*), many of which are presumably from blood plasma. The EDTA-containing extracts were enriched in a protein, COMP, which migrates under reducing conditions as a closely spaced doublet band with an apparent M_r of 120,000 (Fig. 1*a*, *extracts 5–8*, see *arrow*). Similar but weaker bands may also be seen in the preceding low salt extracts (Fig. 1*a*, *extracts 1–4*), indicating that this protein is being slowly released from the tissue also in the absence of EDTA. Further extraction of the tissue residue with buffer containing 4 M guanidine HCl, in addition to the components listed above, released a large number of proteins (Fig. 1*a*, *extracts 9–12*), presumably due to denaturation of these as well as of the insoluble collagen network. Some COMP may be contained in this extract, but it appears that the extraction with EDTA-containing buffer solubilizes a large portion of the total extractable pool of this protein. This conclusion was confirmed by immunoblotting of the first extract obtained with each solvent (Fig. 1*b*), using an antiserum raised against the bovine protein (Hedbom *et al.*, 1992) for identification of COMP. The reduced M_r = 120,000 COMP band was seen most strongly in the first EDTA extract (Fig. 1*b*, *extract 5*). Smaller amounts of this band, together with a strongly stained faster migrating band, were detected in the low salt extract (Fig. 1*b*, *extract 1*). The faster migrating band is likely to represent a soluble degradation fragment of COMP, but has so far not been characterized in detail. The guanidine HCl extract could, by immunoblotting (Fig. 1*b*, *extract 9*), be shown to contain small amounts of COMP. When initial extraction of the tissue is done with buffer containing 0.15 M NaCl, rather than the low salt buffer used in the present study, more protein is released in the washing steps prior to extraction with EDTA-containing buffer, but the basic observation of an increased and selective release by EDTA remains (results not shown).

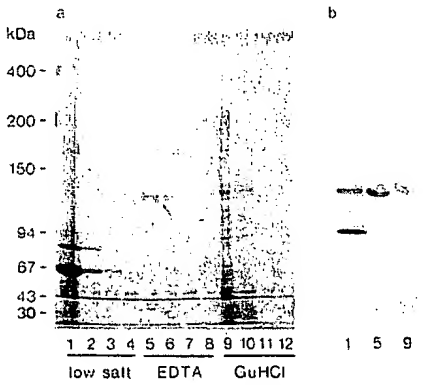

FIG. 1. **Selective extraction of COMP from Swarm rat chondrosarcoma tissue by EDTA-containing buffer.** *a*, samples of sequential extracts were reduced and submitted to SDS-polyacrylamide gel electrophoresis on 4–15% gradient gels. *Lanes 1–4* show consecutive extracts obtained with a low salt buffer at pH 7.4; *lanes 5–8*, following extracts obtained with the low salt buffer containing 10 mM EDTA; and *lanes 9–12*, extracts obtained with the same buffer containing 4 M guanidine HCl and 10 mM EDTA. Reduced COMP polypeptides with an apparent M_r of about 120,000 were enriched in the first EDTA extracts as indicated with the *arrow*. *b*, the migration position of COMP subunits, as well as their relative abundance in the first extract obtained with each solvent, was confirmed by immunoblots. The samples were the same as in *a*, and, after identically performed electrophoresis, proteins were transferred to nitrocellulose and detected with an antibody against bovine COMP. In *extract 1 (low salt)*, a faster migrating band was detected in addition to the M_r = 120,000 COMP subunits. The EDTA extract (5) is enriched in COMP, while only low amounts are found in the guanidine HCl extract (9).

Purification of COMP from EDTA Extracts of Swarm Rat Chondrosarcoma Tissue—The extracts obtained with EDTA-containing buffer, after prewashing the tissue with low salt buffer, were used for further purification of COMP. The extracts were passed over an anion exchange column (DEAE-Sepharose Fast Flow), and bound proteins eluted with a gradient of 0–0.5 M NaCl (Fig. 2*a*). COMP could be detected by SDS-polyacrylamide gel electrophoresis in fractions eluting at salt concentrations of 0.15–0.3 M indicating a moderate negative net charge at the pH of 7.4 which was used. A pool of these fractions was concentrated by ultrafiltration and passed over a gel filtration column of Sepharose CL-4B (Fig. 2*b*). COMP eluted as a rather sharp included peak, and a pool made from this material appeared >90% pure as assessed by SDS-polyacrylamide gel electrophoresis (results not shown). Even so, further purification was done when required by chromatography on an FPLC column of Superose 6 (Fig. 2*c*). COMP eluted in an included peak as well as in the void volume of the column. The latter result indicated a tendency to self-aggregation at an elevated concentration of COMP, in particular as clear differences in the COMP contained in the two pools could not be detected by SDS-polyacrylamide gel electrophoresis (Fig. 3).

Polypeptide Organization and Immunoreactivity—On nonreducing SDS-polyacrylamide gel electrophoresis, purified COMP runs as a doublet band in approximately the same position as that of the reduced M_r = 400,000 laminin A chain (Fig. 3, *lanes 2* and *3*). As nonreduced COMP can be expected to have a more compact structure than a reduced polypeptide, its molecular mass can be assumed to be larger than 400,000. The faster migrating band appears less enriched in EDTA extracts of chondrosarcoma tissue (Fig. 3, *lane 1*) than in purified COMP, which would indicate that it arises from a limited proteolytic degradation occurring during purification, despite the use of protease inhibitors. After reduction, the subunits of COMP are seen as a closely spaced doublet band

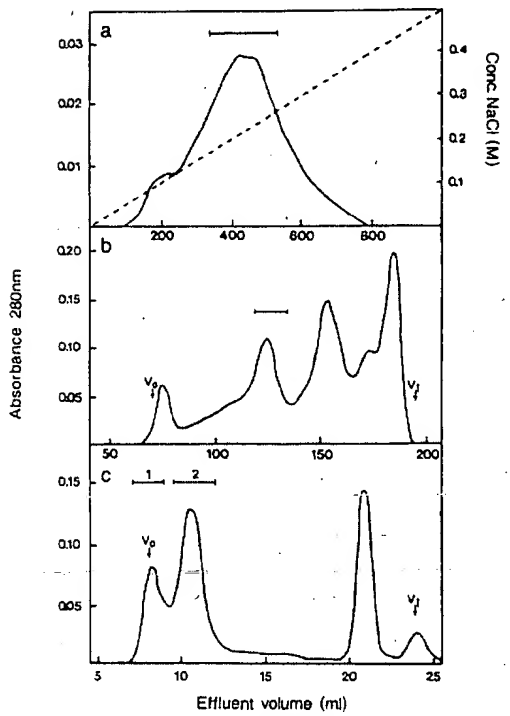

FIG. 2. **Chromatographic purification of COMP from EDTA extracts of Swarm rat chondrosarcoma tissue.** *a*, an EDTA extract of prewashed chondrosarcoma tissue was applied to a column of DEAE-Sepharose Fast Flow and eluted with a linear gradient of 0–0.5 M NaCl in 10 mM Tris/HCl, pH 7.4, containing 2 mM EDTA, 2 mM PMSF, and 2 mM NEM. The *dashed line* indicates the gradient, and the *bar*, the pool made of fractions containing COMP as determined by SDS-polyacrylamide gel electrophoresis of aliquots. *b*, the pool from the the DEAE-Sepharose was concentrated by ultrafiltration and passed over a Sepharose CL-4B molecular sieve eluted with 0.15 M NaCl, 10 mM Tris/HCl, pH 7.4, containing 2 mM EDTA, 2 mM PMSF, and 2 mM NEM. The *bar* indicates the pool made from COMP-containing fractions and V_0 and V_t the void and total volume of the column, respectively. *c*, the COMP pool from the Sepharose CL-4B chromatography was concentrated and rechromatographed on an FPLC Superose 6 HR 10/30 molecular sieve eluted with 0.15 M NaCl, 10 mM Tris/HCl, pH 7.4, containing 10 mM EDTA. Pools of COMP-containing fractions were made as indicated by the bars. It is assumed that PMSF and NEM contribute to the peaks seen close to the V_t of the column.

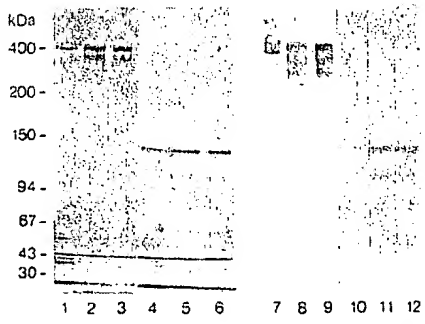

FIG. 3. **Subunit structure and immunoreactivity of COMP.** An EDTA extract (*lanes 1, 4, 7,* and *10*) as well as pools 1 (*lanes 2, 5, 8,* and *11*) and 2 (*lanes 3, 6, 9,* and *12*) of purified COMP after Superose 6 chromatography (Fig. 2*c*) were subjected to SDS-polyacrylamide gel electrophoresis on 4–15% gels. Samples were run under nonreducing (*lanes 1–3* and *7–9*) or reducing conditions (*lanes 4–6* and *10–12*), and proteins were detected either by staining with Coomassie Blue (*lanes 1–6*) or after incubation with an antiserum raised against bovine cartilage COMP (*lanes 7–12*).

with an apparent M_r of 120,000 (Fig. 3, *lanes 4–6*). The faster migrating of these two bands is seen with similar intensity already in the initial EDTA extract (Fig. 3, *lane 4*) indicating that there may be an *in situ* heterogeneity of COMP subunits. An additional band at apparent M_r = 100,000 is seen in the purified COMP and could result from degradation during purification. Under both reducing and nonreducing conditions, some remaining contaminants of apparent M_r below 70,000 are found.

The same samples were also blotted onto nitrocellulose and treated with an antibody raised against COMP (Fig. 3, *lanes 7–12*) isolated from bovine cartilage (Hedbom *et al.*, 1992). This antibody stains rat chondrosarcoma COMP under both nonreducing and reducing conditions, showing the relationship between the two preparations. With antibody staining, some additional material is seen under nonreducing conditions, running above the major COMP bands. This staining is particularly prominent in the EDTA extract (Fig. 3, *lane 7*) and could represent aggregates of COMP which are largely removed during purification. An antiserum raised against the slower migrating of the apparent M_r = 400,000 bands of rat COMP, cut out from an SDS-polyacrylamide gel, reacts with this high M_r material as well as with the faster migrating band appearing in purified COMP (results not shown). This result indicates that all these components contain the same epitopes and that the staining is not due to contaminating antibodies against unrelated high M_r proteins. Under reducing conditions, both the M_r = 120,000 band and the M_r = 100,000 degradation product were stained by the antibodies (Fig. 3, *lanes 10–12*). From the width of the band it appears that both components of the M_r = 120,000 doublet were stained, but the doublet band was not resolved in the immunoblots.

Electron Microscopy—Molecules of COMP were visualized after spraying from glycerol/buffer mixtures and rotary shadowing (Fig. 4) and after adsorption to carbon films and negative staining (Fig. 5). By both techniques, uniform fields with particles of complex appearance were obtained. In most molecules, five globular units connected by thin flexible strands to a central domain could be distinguished. The latter was most clearly seen after negative staining as a short cylinder with dimensions of 3.3 ± 0.4 nm in diameter and 7.7 ± 0.6 nm in length. Globular domains appeared with a diameter of 4.8 ± 0.7 nm in negatively stained images. These dimensions were enhanced by about 2.5 nm after rotary shadowing due to decoration by metal crystallites (Engel and Furthmayr, 1987). In addition, the connecting strands appeared unrealistically thick by this technique. From negatively stained particles, their diameter was estimated to be 1.2 nm. The contour lengths of well resolved strands were traced.

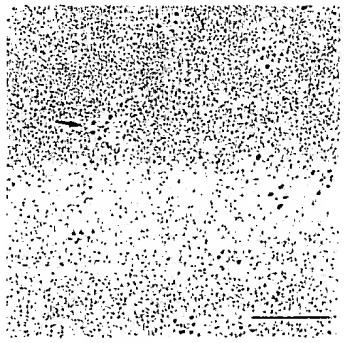

FIG. 4. **Structure of COMP as revealed by glycerol spraying/rotary shadowing electron microscopy.** A representative field of five-armed molecules (*arrow*) is shown. The *bar* represents 200 nm.

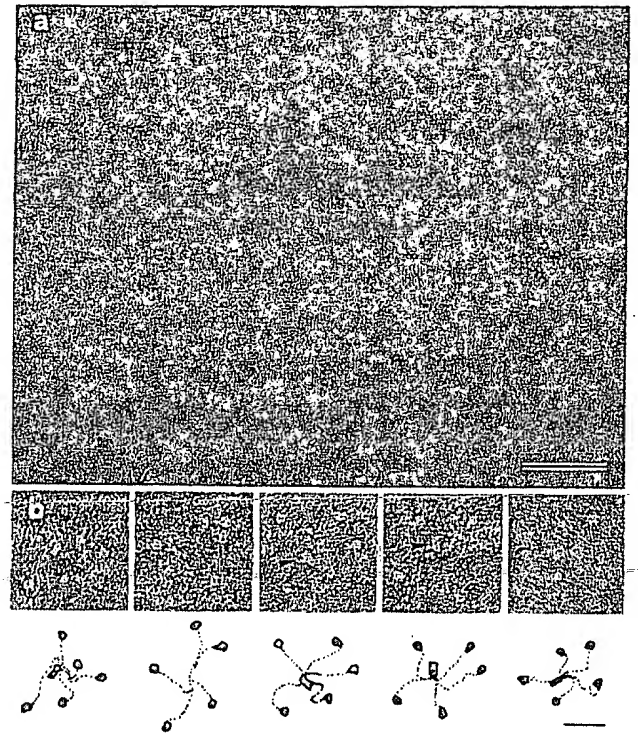

FIG. 5. **Structure of COMP as seen by negative staining and electron microscopy.** *a*, representative field of COMP particles obtained after negative staining with uranyl formate. The *bar* represents 100 nm. *b*, selected particles at higher magnification with interpretative drawings. In some cases, intersection regions of the connecting strands are indicated with *arrowheads*. The *bar* represents 25 nm.

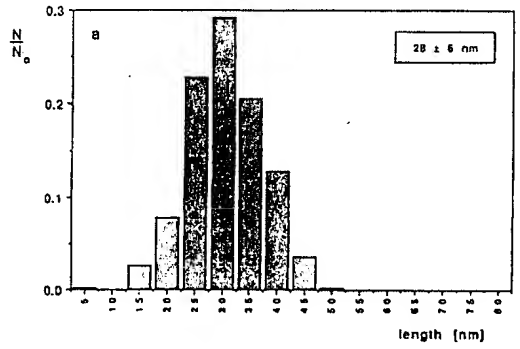

FIG. 6. **Length distribution of the thin connecting strands of COMP measured from the center of the globular region to the intersection of the chains.** Measurements were made on about 400 particles. N/N_0 denotes the fraction of molecules with a length of the strands within an interval of ±5 nm. The average value ± S.D. is given in the diagram.

No significant differences in the length distribution of negatively stained and shadowed particles were observed (Fig. 6), and an average length of 28 ± 6 nm was evaluated. No difference in length was seen between samples sprayed in the presence of calcium or after incubation with EDTA.

DISCUSSION

Extraction of COMP from chondrosarcoma tissue was greatly facilitated by the presence of EDTA in the solvent. This shows that anchorage of this protein in the cartilage-like matrix is dependent on divalent cations, either directly or by divalent cations inducing a conformation in COMP and/or an interaction partner favoring binding. COMP has been shown to be localized to the cartilage matrix by immunohistochemistry (Fife *et al.*, 1985; Franzen *et al.*, 1987; Hedbom *et al.*, 1992). This, together with the present results, indicates that it is a cartilage matrix protein less tightly bound than most others and showing a dependence on concentration and composition of its ionic environment for anchorage.

The selective solubility in EDTA-containing buffer made a purification of the native protein feasible. In the initial EDTA extract it is the major protein component. In addition to COMP, two proteins of M_r = 30,000–40,000 were selectively extracted in this step (Fig. 1*a*). Chromatography on DEAE-Sepharose (Fig. 2*a*) showed that COMP has a negative net charge at physiological pH which was useful in concentrating the protein as well as removing more basic proteins and co-extracted proteoglycans. On gel filtration, COMP showed a tendency for aggregation, in particular when applied at high concentrations (Fig. 2*c*). It is not yet known if this self-association is of physiological relevance. Some degradation occurred during purification, presumably due to the presence of endogenous proteases that were not efficiently inhibited by the protease inhibitors added. This was detected by the appearance of a second band, migrating just below the original COMP in nonreducing SDS-polyacrylamide gel electrophoresis (Fig. 3, *lanes 2, 3* and *8, 9*), and a fraction of the reduced polypeptides migrating with an apparent M_r of 100,000 rather than the original 120,000 (Fig. 3, *lanes 5, 6* and *11, 12*). As the degradation affected a relatively small proportion of the total protein, it is unlikely to influence the interpretation of electron microscopy images. The rather distinct degradation pattern indicates the presence af a site within each polypeptide which is particularly sensitive to proteolysis. Among the M_r = 120,000 subunits, two very tightly spaced components can be discerned (Fig. 3, *lanes 4–6*). As these can be seen in the initial EDTA extract, they are less likely to be due to proteolytic degradation and could arise through differential glycosylation or alternative splicing.

In rotary shadowing, the majority of molecules showed a structure with five arms terminating in a globular domain (Fig. 4). The arms had an average length of 28 nm and displayed no clear signs of heterogeneity (Fig. 6). Higher resolution was achieved by electron microscopy after negative staining (Fig. 5). By this technique it was revealed that the arms are joined in a central cylindrical structure with 3.3 nm in diameter and 7.7 nm in length. The globular domains have a diameter of 4.8 nm in those images from which their molar mass of 30,000 to 45,000 can be estimated (Engel and Furthmayr, 1987). On the basis of these observations, a bouquet-like structure with five flexible arms terminated by globular

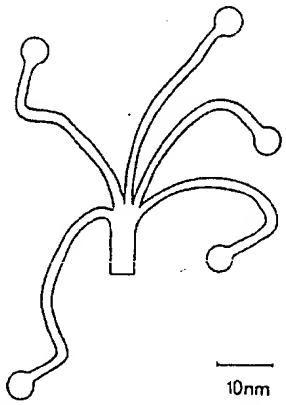

FIG. 7. **Model of cartilage oligomeric matrix protein.**

heads is suggested for COMP (Fig. 7). Similar structures have been described for a variety of oligomeric proteins, including subcomponent C1q of the first component of complement, surfactant protein A, mannan-binding protein (reviewed by Thiel and Reid, 1989), and thrombospondin (Lawler and Hynes, 1987). The arms in the first three mentioned proteins are collagen triple helices, which although comparable in diameter appear more rigid in electron micrographs than the frequently bent arms of COMP. In this respect, COMP closely resembles thrombospondin, whose arms are composed of Cys-rich domains (Lawler *et al.*, 1985; Lawler and Hynes, 1987). The length of the arms in COMP matches that determined for thrombospondin in the presence of calcium, but is 10 nm shorter than in the calcium-free form. In contrast to COMP, thrombospondin is a trimeric structure.

With C1q, the importance of multivalent interactions of its globular heads with the Fc regions in IgG clusters has been demonstrated (Tschopp *et al.*, 1980). Likewise, it was shown for mannan-binding protein, which exists in trimeric to hexameric forms, that carbohydrate ligands are only recognized by penta- and hexamers (Lu *et al.*, 1990). Although the nature of the target sites differ considerably for the various proteins and is unknown for COMP, a common functional feature of all bouquet-like proteins is most likely the binding to one or several interaction partners.

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